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ORIGINAL ARTICLE

Induction and treatment of anergy in murine leprosy

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SUMMARY

Leprosy is a disease consisting of a spectrum of clinical, bacteriological, histopathological and immunological manifestations. Tuberculoid leprosy is frequently recognized as the benign polar form of the disease, while lepromatous leprosy is regarded as the malignant form. The different forms of leprosy depend on the genetic and immunological characteristics of the patient and on the characteristics of the leprosy bacillus. The malignant manifestations of lepromatous leprosy result from the mycobacterial-specific anergy that develops in this form of the disease. Using murine leprosy as a model of anergy in this study, we first induced the development of anergy to *Mycobacterium lepraemurium* (MLM) in mice and then attempted to reverse it by the administration of dialysable leucocyte extracts (DLE) prepared from healthy (HLT), BCG-inoculated and MLM-inoculated mice. Mice inoculated with either MLM or BCG developed a robust cell-mediated immune response (CMI) that was temporary in the MLM-inoculated group and long-lasting in the BCG-inoculated group. DLE were prepared from the spleens of MLM- and BCG-inoculated mice at the peak of CMI. Independent MLM intradermally-inoculated groups were treated every other day with HLT-DLE, BCG-DLE or MLM-DLE, and the effect was documented for 98 days. DLE administered at a dose of 1.0 U (1×10^6 splenocytes) did not affect the evolution of leprosy, while DLE given at a dose of 0.1 U showed beneficial effects regardless of the DLE source. The dose but not the specificity of DLE was the determining factor for reversing anergy.

Keywords
anergy, BCG, leucocyte extracts, murine leprosy, *Mycobacterium lepraemurium*

Leprosy is an ancient disease that still exists today although with a lessened incidence in some parts of the world (WHO 2013). The disease is caused by *Mycobacterium leprae*, an intracellular parasite of macrophages. One of the hallmarks of the disease is its bipolar manifestation across a spectrum of intermediate forms. Tuberculoid leprosy (TT) is the benign polar form of the disease, and lepromatous leprosy (LL) is the malignant form (Ridley & Jopling 1966). The form and severity of the disease seem to depend on both the virulence of *M. leprae* (Han *et al.* 2008, 2009) and the characteristics of the host because highly susceptible individuals develop LL, while more resistant individuals develop TT (Marquet & Schurr 2001). The progression of leprosy in LL occurs because the host immune response is impaired as a result of mechanisms that are still not well understood, giving rise to *M. leprae*-specific cellular anergy (Rojas-Espinosa 2007). Anergy does not occur in TT, in which the disease evolves less aggressively and often disappears in a spontaneous manner. Anergy in LL seems to implicate triggering by microbial components and impairment of pathways leading to IL-2 production, a key proliferation factor for T cells (Dagur *et al.* 2010, 2012). We hypothesized that because it is a microbial-induced T-cell defect, anergy might be reversed or modulated by the use of immunomodulators such as transfer factor (TF) from sensitized donors.

Transfer factor has a long history and has been widely used in the treatment of several diseases (Berrón *et al.* 2007; Viza *et al.* 2013). It is obtained from sensitized lymphoid cells and has the ability to transfer immunity from sensitized to non-sensitized individuals. Due to its reported beneficial effect in

both infectious and non-infectious diseases, TF has been considered an immunomodulator (Wilson & Fudenberg 1979). In leprosy, the administration of TF has provided results ranging from null to encouraging, although with no curative outcomes (Bullock *et al.* 1972; Saha *et al.* 1975; Hastings & Job 1978; Faber *et al.* 1979; Leser *et al.* 1980). However, the administration of TF to patient populations that are heterogeneous with respect to race, sex, age, length of disease, treatment and associated complications such as leprosy reactions and disease sequelae has made it difficult to evaluate the potential utility of this substance. Nevertheless, leprosy has an approximate equivalent in murine leprosy. Murine leprosy, caused by *Mycobacterium lepraemurium*, *is not the same disease as human leprosy,* but it shares with it several characteristics (Rojas-Espinosa 2009), among which anergy is the most relevant characteristic for this study. In addition, murine leprosy is in itself a relevant disease because *M. lepraemurium* (MLM) is the aetiologic agent of cat leprosy, a disease with significant prevalence in Australia and New Zealand, as well as in other countries such as Canada, the United States, France, Germany, and England (Makik *et al.* 2002). Human and murine leprosy evolve with the development of anergy, or more exactly, anergy is the main reason for the progression of leprosy. Thus, taking advantage of the murine leprosy model, a disease that can be fully controlled in relation to the host (syngeneic strains offer the opportunity to work with homogeneous mouse populations) (Closs & Haugen 1974), time of infection and dose of the bacilli administered, among others, it is possible to evaluate the effect of TF and other substances, including novel anti-leprosy drugs, on the progression of the disease. Mice with an intermediate susceptibility to murine leprosy develop an initial disease with characteristics of TT that then switches to the lepromatous (anergic) form of the disease. *M. bovis* BCG, in contrast, produces a self-healing disease because it elicits a long-lasting, protective, cell-mediated immune response. Therefore, in this study, we analysed the effect of MLM-specific and non-specific (BCG) transfer factors on the progression of murine leprosy and compared the results with those obtained from a group of mice treated with a combination of rifampicin and clofazimine. Because the substances used in this study are actually whole dialysable leucocyte extracts (DLE), we will use this term instead of transfer factor (TF), the term originally provided by Lawrence (1955) to this immunity-transferring substance.

Materials and methods

Chemicals

Unless otherwise indicated, most chemicals were purchased from Sigma-Aldrich Chemical Co (St. Louis MO, USA).

Ethical approval

Research approved by the Comisión de Etica en Investigación de la Escuela Nacional de Ciencias Biológicas under the code CEI-ENCB 016/2014.

Bacilli

Mycobacterium lepraemurium, Hawaii strain, was isolated from the spleens and livers of mice bearing a 4-month infection with *M. lepraemurium* by differential centrifugation on gradients of sucrose, KCl and percoll as described by Mendoza-Aguilar *et al.* (2012). *Mycobacterium bovis* BCG was expanded from the Glaxo 1077 BCG vaccine (Paris, France) cultured in Middlebrook 7H9 broth supplemented with 5% Middlebrook OADC enrichment, which were both from DIFCO laboratories, Detroit MI, USA.

Mice

Female NIH mice weighing 20–22 g were purchased from Birmex (Mexico City) and handled under the Mexican Norm for the human handling of experimental animals (NOM-029-200-1995).

Cell-mediated immunity

A group of 36 mice was inoculated with 20×10^6 *M. lepraemurium* (MLM) by the intraperitoneal route, and a similar group was inoculated with 20×10^6 *M. bovis* BCG (BCG) via the same route. At weekly intervals three mice in each group were anaesthetized, killed by heart puncture exsanguination and underwent spleen removal. Spleen cells were separated by gently pressing small spleen fragments with the back of a syringe plumber on a cell sieve (Falcon) using DMEM as the suspension medium. Cell numbers were adjusted to 5×10^6 cells/ml, and aliquots of 0.1 ml (0.5×10^6 cells) per well were placed in 96-well culture plates (Nunc, Roskilde, Denmark) in quadruplicate. Cells were stimulated either with nil, 10 μg/10 μl of MLM- or BCG-soluble extracted proteins, or with 0.05 μg/10 μl of concanavalin A (ConA). Cultures were maintained for 3 days at 37°C in a humid atmosphere containing 5% CO_2. Twelve hours before harvesting, 20 μl of Blue Alamar (Biosource, Invitrogen, CA, USA) was added to each well, and the fluorescence emitted by the reduced product was registered using a Fluoroskan Ascent FL (Thermo Fisher Scientific Inc., Walthman MA, USA).

Antibody response

Anti-mycobacterial antibodies in the serum of BCG- or MLM-inoculated animals were measured using an enzyme-linked immunosorbent assay (ELISA) performed as described elsewhere (Wek-Rodriguez *et al.* 2007).

In vivo *macrophage activation*

Macrophage activation was assessed through the expression of β-galactosidase activity following a procedure described elsewhere (Rojas-Espinosa *et al.* 1988).

Nitric oxide production

Peritoneal macrophages were collected from the same mice used for cell proliferation. The detailed procedure for macrophage collection has been described in detail by Aguilar *et al.* (2013). For the assay, 0.5×10^6 cells per well in 96-well plates were stimulated with nil, BCG or MLM bacilli (MOI 10:1), or LPS (0.05 μg/10 μl/well). Cultures were maintained for 24 h at 37°C and 5% CO_2 after which 50 μl of supernatant was admixed with 50 μl of Griess reagent in a fresh plate, and colour development was read at 540 nm using an ELISA reader (Labsystem, Multiskan Plus, Helsinki, Finland) (Green *et al.* 1982). Griess reagent is 1% sulphanilamide in water and 0.1% N-1-naphthyl ethylenediamine.2HCl in 2.5% phosphoric acid (vol. to vol.).

Dialysable leucocyte extracts (DLE)

DLE, the source of transfer factors (TFs), were prepared from the spleens of healthy mice, or from mice inoculated with MLM or BCG at their peak lymphoproliferative response to the corresponding mycobacterial antigens. The procedure included the preparation of spleen cell suspensions as described above, the counting and adjustment of the cells to equivalent concentrations and the disruption of the cells through 10 cycles of freezing (dry ice acetone) and thawing (35°C). The resulting disrupted suspensions were dialysed against pyrogen-free sterile physiological saline solution (PSS) for 48 h at 4°C at a ratio of 10^7 cells per ml, and then the dialysis solution was collected, passed through a sterile 0.2-μm Millipore membrane, divided into 1.0-ml aliquots and kept frozen at −20°C until used (one unit of DLE corresponded to the amount of protein extracted from 1×10^6 splenocytes).

Infection and treatment

Intradermal inoculation of mice with MLM. NIH albino, 5-week-old mice were intradermally inoculated with 2×10^6 bacilli in 10 μl of PSS in the abdominal region. Five weeks after inoculation, most animals developed incipient but distinctive leprosy lesions that progressed steadily without treatment to reach large dimensions and cause systemic infection over the long term.

Treatment of MLM-infected mice. Mice bearing a 5-week intradermal infection with MLM were separated into 8 groups of five mice each. Each group received either PSS (group G1), 1.0 U of DLE from healthy mice (HLT-DLE) (G2), 0.1 U of HLT-DLE (G3), 1.0 U of DLE from mice inoculated with MLM (MLM-DLE) (G4), 0.1 U of MLM-DLE (G5), 1.0 U of DLE from mice inoculated with BCG (BCG-DLE) (G6), 0.1 U of BCG-DLE (G7) or a cocktail of rifampicin (7.5 mg/kg) and clofazimine (1.6 mg/kg) (group G8). Treatment was administered intragastrically every other day for 98 days.

Analysis

Morphologic analysis. Mice were killed with an excess of anaesthetic at the end of the experiment. The skin was removed from the injection site and processed for paraffin sectioning following a standard procedure. Four-micron-thick sections were prepared from each skin sample and stained using the Ziehl–Neelsen, haematoxylin–eosin and Masson's techniques. Histopathological descriptions of the lesions were based on these stains.

Image analysis. Tissue sections were analysed under the microscope (Nikon Eclipse E800, Tokyo, Japan), and images

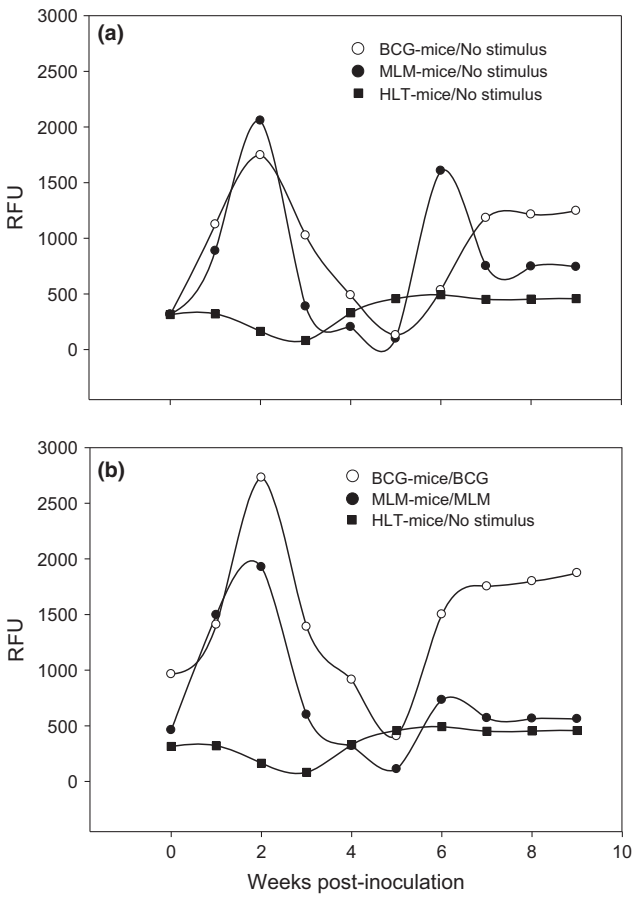

Figure 1 Panel (a): Spontaneous proliferation of spleen cells from mice inoculated with BCG (BCG-mice) or MLM (MLM-mice). Panel (b): Mycobacterial-induced proliferation of spleen cells from mice inoculated with BCG or MLM. The spontaneous proliferation of spleen cells from healthy mice (HLT-mice) is also shown. Standard deviations are omitted to avoid overcrowding of the images. Alamar blue fluorescence assay. RFU indicates relative fluorescence units ($P < 0.001$ *vs.* healthy mice at the specified points).

were obtained at several magnifications. The extents of the bacilli and cell infiltrates were analysed using IMAGE software (Wayne Rasband NIH, Bethesda, MD, USA), and the results were reported as pixels/mm^2.

Results

Contrary to MLM, BCG induces a long-lasting cell-mediated immune response

Both BCG- and MLM-inoculated mice developed a cell-mediated immune response, and this immune state was observed even in the absence of any *in vitro* stimulation. The peak of this 'spontaneous' lymphocyte proliferation was observed at 2 weeks postinoculation in both BCG- and MLM-inoculated mice. By 6 weeks postinoculation, a second peak of spontaneous lymphoproliferation was observed in the MLM-inoculated group but not in the BCG-inoculated group; this is a meaningful finding because this proliferating cell population might be the one that Mathew *et al.* (1984) described with suppressor activity, a point that deserves further investigation in our model of murine leprosy (Figure 1a). The *in vitro* antigen-specific challenge of spleen cells from BCB- and MLM-mice did not change this response pattern but increased the levels of proliferation to some extent excluding the spontaneous proliferation peak observed at 6 weeks in the MLM-inoculated group, which diminished considerably (Figure 1b). After the peaks of spontaneous- and mycobacterial-triggered lymphoproliferation at 2 weeks in both groups of mice, the proliferative response was exhausted, reaching minimal levels at 5 weeks postinoculation. Subsequently, it recovered in the BCG-inoculated group but not in the MLM-inoculated group ($P < 0.001$), suggesting the development of cellular anergy in the latter group.

Beta-galactosidase activity correlates with the mycobactericidal activity of macrophages

Macrophage activation in the spleen of the inoculated mice was assessed by the beta-galactosidase (β-Gal) histochemical stain (Rojas-Espinosa *et al.* 1988). When activated, macrophages possess an elevated content or activity of lysosomal enzymes. β-Gal is a good marker of macrophage activation because it produces a deep blue–indigo colour that contrasts well with the fuchsia of acid-fast bacilli (AFB) in the Ziehl–Neelsen stain (Figure 2).

Different profiles of β-Gal expression were observed in the spleens of mice inoculated with BCG or MLM. The peak activity appeared between weeks 3 and 4 postinoculation in both the BCG- and MLM-inoculated groups, after which significant differences were observed. In BCG-infected mice, the peak of maximal macrophage activation occurred by 3 weeks postinoculation, and then vanished by week 5 and remained undetectable until the end of the experiment. The number of bacilli, however, showed a modest peak that was coincident with the peak of maximal β-Gal activity and

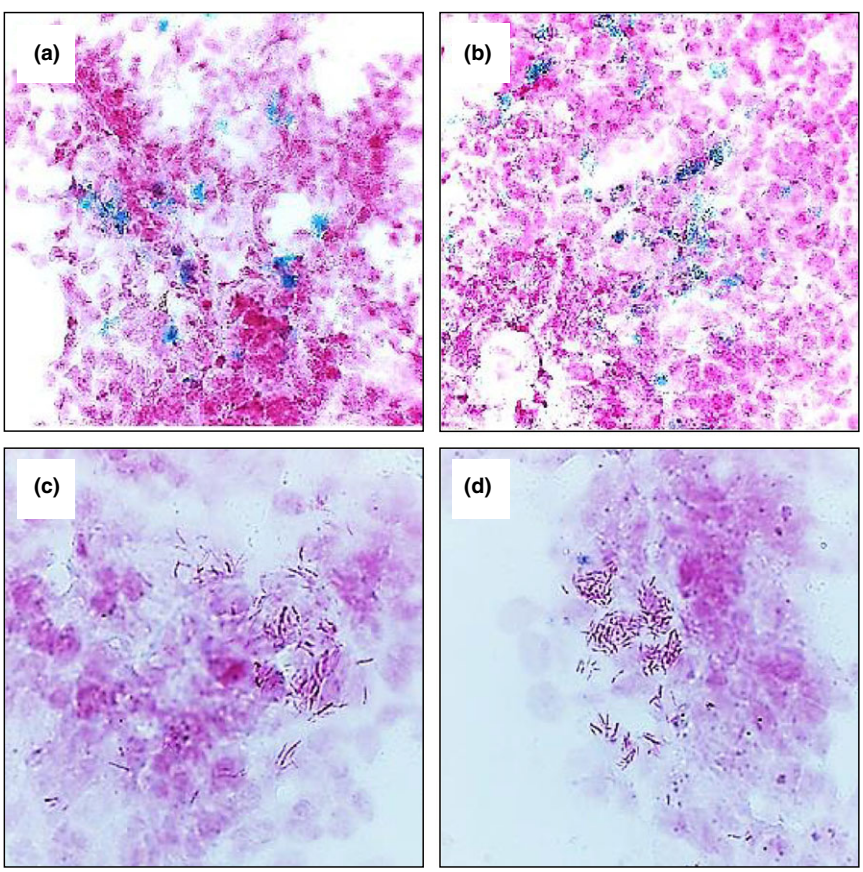

Figure 2 Macrophage activation in the spleens of mice inoculated with MLM at 2 (Panels a and b) and 7 (Panels c and d) weeks of inoculation. Spleen cells were first stained for beta-galactosidase (β-Gal) and then for acid-fast bacilli; notice that β-Gal activity disappeared completely by 7 weeks postinoculation, while the number of bacilli reached considerable numbers (β-Gal images taken at 40 × 10X; acid-fast bacilli taken at 100 × 10X).

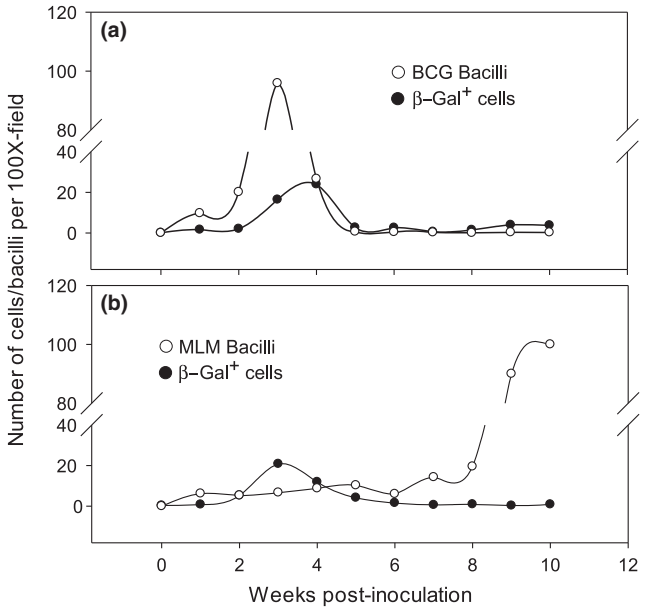

Figure 3 Panel A: Macrophage activation (B-Gal expression) in the spleens of mice intravenously inoculated with BCG, and the number of bacilli in the spleens of the animals during 10 weeks of inoculation. Panel B: Macrophage activation (β-Gal expression) in the spleens of mice inoculated with MLM, and number of MLM bacilli in the spleens of the animals during 10 weeks of inoculation (average values from 3 fields; 100 bacilli are actually 100 or more bacilli). Standard errors are not shown to avoid overcrowding of the image.

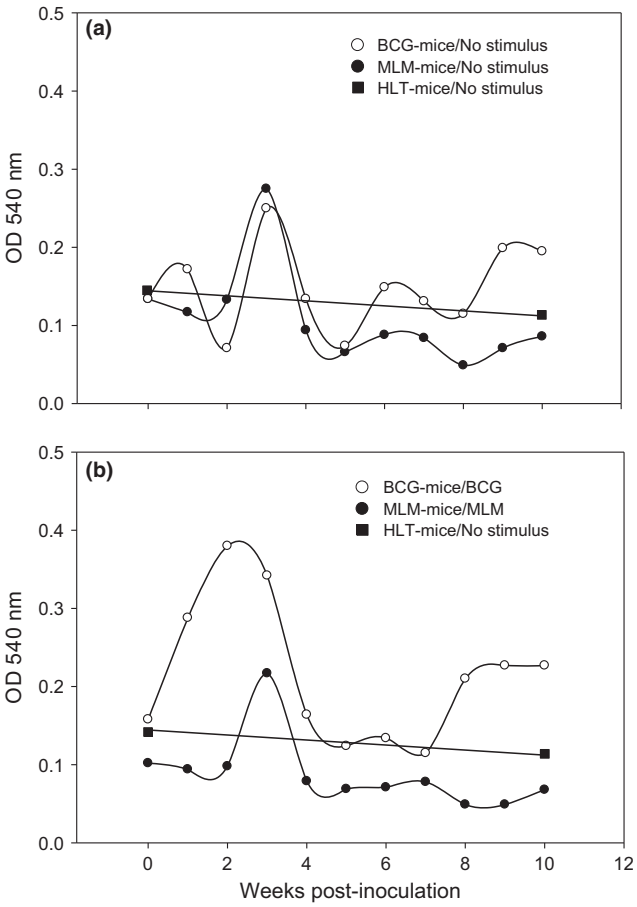

Figure 4 Panel (a): Spontaneous nitric oxide production by peritoneal macrophages from mice inoculated with BCG (BCG-mice) or MLM (MLM-mice) without introducing external antigen. Panel (b): Nitric oxide production by macrophages from mice inoculated with BCG or MLM in response to *in vitro* stimulation with BCG or MLM. The spontaneous nitric oxide production by macrophages from healthy mice is shown as a fit line. The results represent averages from 3 mice, and experiments were performed in triplicate. No standard error values are shown to avoid overcrowding of the images.

then rapidly diminished to undetectable levels by 5 weeks postinoculation to the end of the experiment. BCG clearly induced a strong early macrophage activation that efficiently cleared the microorganism (Figure 3a). In the MLM-inoculated group, β-Gal expression also peaked at 3 weeks after inoculation but mostly disappeared by week 5 until the end of the experiment. At 3 weeks postinoculation, no bacilli were observed, but from this time on, the number of bacilli increased proportionally to the time of inoculation until they were too many to count at 9 weeks. This finding suggests that activated macrophages were able to contain the replication of MLM until they become inactivated by weeks 5–6, and then the bacilli grew freely to reach uncountable numbers (Figure 3b).

BCG but not MLM induces sustained production of nitric oxide by macrophages

Peritoneal macrophages from BCG- or MLM-inoculated mice spontaneously (*i.e.* in the absence of *in vitro* stimulation) produced nitric oxide (NO) in amounts that peaked on week 3 postinoculation, after which NO production decreased to negligible levels by week 5. Subsequently, the spontaneous production of NO recovered to significant levels in the BCG-inoculated mice but not in the MLM-inoculated mice. Macrophages from healthy non-infected mice released steady background levels of NO (Figure 4a). *In vitro* stimulation of macrophages with BCG or MLM bacilli significantly increased the production of NO by week 3 in the BCG-inoculated mice but not in the MLM-inoculated mice. Subsequent NO production remained at background levels in the MLM-inoculated mice but recovered to significant levels in the BCG-inoculated mice (Figure 4b).

Contrary to MLM, BCG induces an early but contained antibody response

Mice inoculated with BCG produced anti-BCG antibodies since very early in the infection. The level of antibodies was already high by week 2 postinoculation and continued rising to peak at weeks 5–6, after which it steadily diminished until week 9 when the study was ended. In contrast, mice

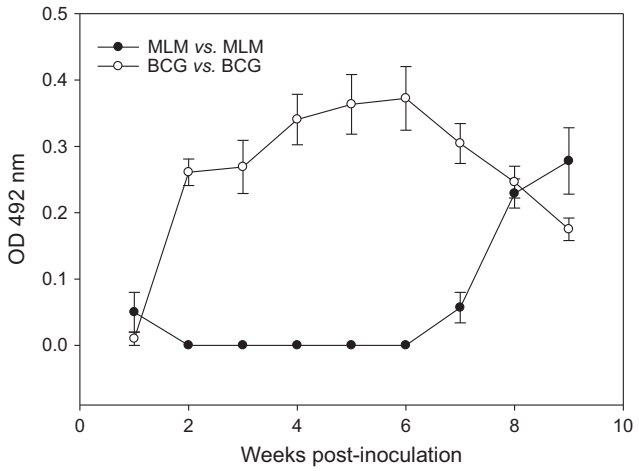

Figure 5 Level of antibodies in the sera of mice inoculated with BCG (BCG) or *Mycobacterium lepraemurium* (MLM) by an ELISA using soluble antigens from BCG or MLM. The results represent averages from 3 mice in each group, and the experiments were performed in triplicate. OD_{492} is the optical density at 492 nm.

inoculated with MLM did not show evidence of antibody production until week 7, following which the levels of antibody increased progressively until the end of the study at week 9 (Figure 5).

Evolution of the lesions of murine leprosy

The size of the MLM-induced lesions in mice subjected to treatment was recorded during the course of infection. Two evolution profiles were observed depending on the amount and source of DLE used for treatment. Compared with the MLM-infected untreated group, the administration of DLE from any source at the (high) dose of 1.0 U did not show a beneficial effect on the development of murine leprosy up to 14 weeks when the experiment was dismissed (Figure 6a). The effect of DLE was different when the animals were treated with the (low) dose of 0.1 U; mice receiving DLE from BCG-infected or healthy mice displayed a clear control of the disease (Figure 6b). However, the best result was again observed with the rifampicin and clofazimine cocktail, which, as expected, had a clear curative effect. The images in Figure 7 correspond to the animal with the largest lesion development within each group.

Bacillary load

The semi-quantitative assessment of the bacillary load in each group of mice is illustrated in Figure 8. Quantitation of the bacilli was attained using the IMAGE J program, and the units are provided in (red) pixels. The results show the beneficial effect of DLE administered at the low dose (0.1 U) but not at the high (1.0 U) dose. The less efficient

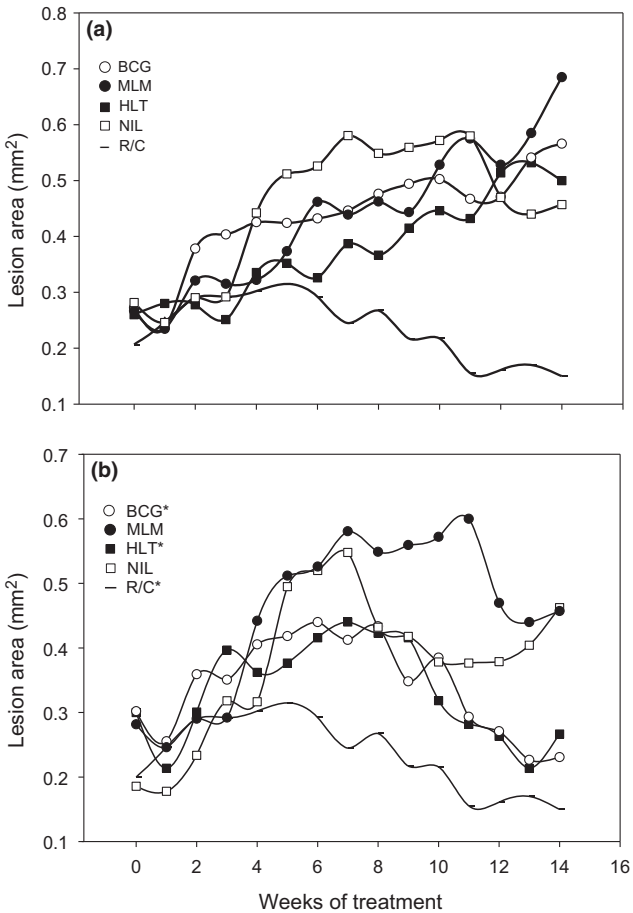

Figure 6 Effect of diverse treatments on the size of murine leprosy lesions over 14 weeks of intradermal inoculation of mice with MLM (MLM-IDI). Panel (a): treatment with 1.0 U DLE; Panel (b): treatment with 0.1 U DLE. NIL indicates untreated MLM-IDI mice, MLM is MLM-IDI treated with DLE from MLM-inoculated mice, BCG is MLM-IDI mice treated with DLE from BCG-inoculated mice, HLT is MLM-IDI mice treated with DLE from healthy mice, R/C is MLM-IDI mice treated with a combination of rifampicin and clofazimine. The results represent averages from 3 mice. No standard errors are shown to avoid masking the response curves (*$P \leq 0.001$ at 12–14 weeks *vs.* NIL).

DLE given at the low dose was the one prepared from MLM-inoculated mice, and again, the anti-leprosy drugs were among the most efficient treatments.

Histopathological findings

The skin lesions from each mouse were excised and processed for paraffin sectioning, and the 4-μm-thick sections were stained for general histology (haematoxylin-eosin), acid-fast bacilli (Ziehl–Neelsen) and collagen (Masson's). The main changes are described below, and the images shown in Figure 9 correspond to the bacilli content of the granuloma fraction in the most affected mouse within each group.

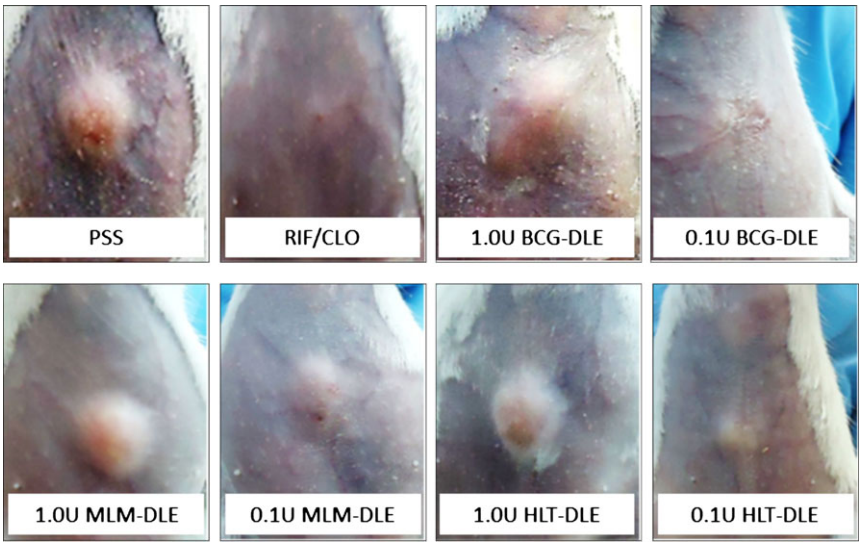

Figure 7 External appearance of MLM-induced skin lesions in mice after 3 months of treatment with nil (untreated), a combination of rifampicin and clofazimine (RIF-CLOF), 1.0 or 0.1 U of dialysable leucocyte extract (DLE) from BCG-inoculated mice (BCG-DLE), 1.0 or 0.1 U of DLE from MLM-inoculated mice (MLM-DLE), or 1.0 or 0.1 U of DLE from healthy mice (HLT-DLE). Images correspond to the animal in each group with maximal lesion development.

Histopathological changes in untreated MLM-infected mice

This group of mice exhibited the greatest histopathological changes, including marked tissue disorganization and an extensive granuloma fraction with chronic inflammatory infiltrates consisting of macrophages, mononuclear cells and foamy cells, with foci of polymorphonuclear (PMN) infiltrates and scattered acellular necrotic areas surrounded by collagen deposition. Cells in the mononuclear infiltrate, including foamy cells, were highly bacilliferous (Figure 9a).

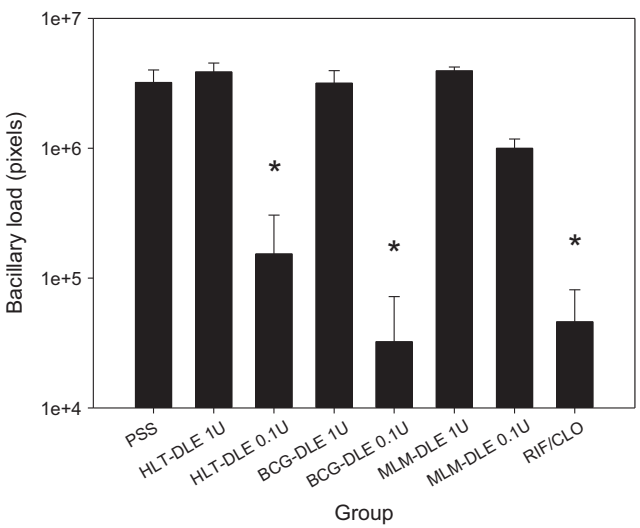

Figure 8 Bacillary load (AFB) (pixel measurement) in the lesions of mice that were intradermally inoculated with MLM (MLM-IDI mice) and treated for 98 days with physiological saline solution (PSS), 1.0 or 0.1 U of DLE from healthy mice (HLT-EDL), 1.0 or 0.1 U of DLE from BCG-inoculated mice (BCG-DLE), 1.0 or 0.1 U of DLE from MLM-inoculated mice (MLM-DLE), or a cocktail of rifampicin and clofazimine (RIF/CLOF). The results represent averages from 3 mice (*$P < 0.001$ *vs.* PSS).

Histipathological changes in MLM-infected mice treated with DLE from MLM-inoculated mice (MLM-DLE)

A representative image of a skin lesion in a MLM-infected mouse treated with 1.0 U of MLM-DLE shows an extended bacilliferous granuloma fraction made of mononuclear cells surrounding discrete foci of PMN infiltrates that were mostly degenerated and intralesional collagen deposition (Figure 9c). A comparative image of a skin lesion from a mouse treated with 0.1 U of MLM-DLE shows significant changes in the bacilli-containing granuloma fraction that has a reduced size but is still widespread (Figure 9d). The remaining bacilli-containing infiltrates consist of necrotic PMN confined to the centre of the lesion.

Histopathological changes in the skin of MLM-infected mice treated with DLE from BCG-inoculated mice (BCG-DLE)

The image in Figure 9(e) corresponds to a skin lesion of a mouse treated with 1.0 U of BCG-DLE. It shows a highly disorganized tissue with a large bacilliferous granuloma fraction consisting mainly of mononuclear cells with some necrotic acellular zones surrounded by foamy cells, many of which contain bacilli, and collagen deposition in the extracellular matrix surrounding the infiltrates. For comparison, Figure 9(f) shows a skin lesion from a mouse treated with 0.1 U of BCG-DLE. A drastic reduction of the dermal bacilli-containing granuloma is evident and has been replaced by extracellular matrix made of granulation tissue. In this image, a deep dermal infiltrate consisting of degenerating PMN cells shows necrotic acellular foci and the remaining bacilli.

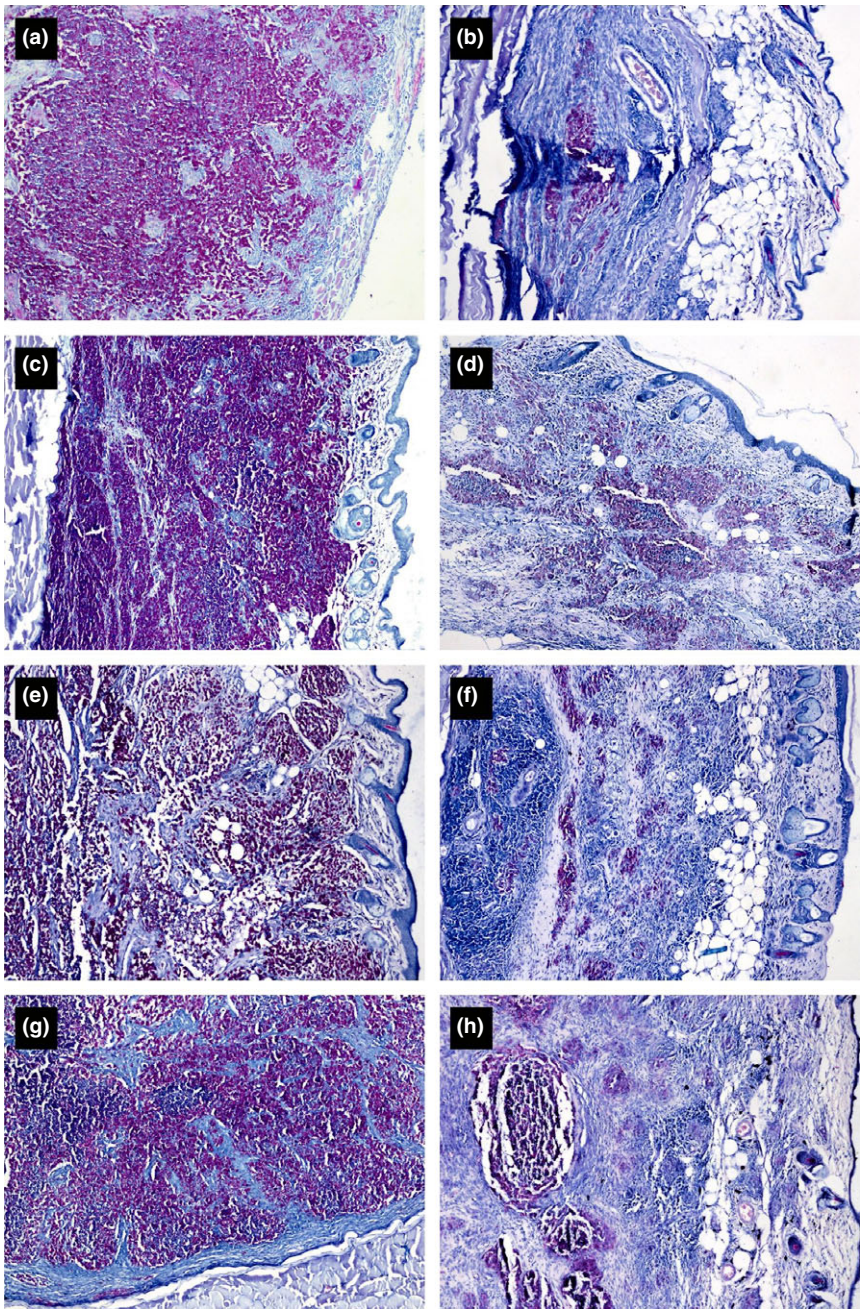

Figure 9 Treatment of mice with murine leprosy with physiologic saline solution, PSS (a), a combination of rifampicin and clofazimine (b), 1.0 U (c) or 0.1 U (d) of DLE from MLM-inoculated mice, 1.0 U (e) or 0.1 U (f) of DLE from BCG-inoculated mice, or 1.0 U (g) or 0.1 U (h) of DLE from healthy (HLT) mice. The granuloma fraction (MLM-loaded macrophages) appears as red-fuchsia, and the tissue is blue (Ziehl–Neelsen and toluidine blue stains, 40 × 10X).

Histopathological changes in MLM-infected mice treated with DLE from healthy mice (HLT-DLE)

An image of the skin lesion in a mouse from the group treated with 0.1 U of HLT-DLE is illustrated in Figure 9(g). It shows a highly disorganized tissue with an extensive mononuclear granulomatous infiltrate, multiple necrotic acellular foci, large amounts of acid-fast bacilli, and bacilli-containing foamy cells, fibroblasts and collagen deposition peripheral to the mononuclear infiltrates, suggesting an ongoing regenerative process. Figure 9(h) shows a mouse treated with 0.1 U of HLT-DLE. The granuloma fraction shows a considerable reduction of the extension with scattered foci of infiltrates consisting of peripheral mononuclear cells and central necrotic areas with degenerated PMNs (piocytes) and bacilli; perilesional foamy cells and intralesional collagen deposition are still present.

Discussion

Human leprosy is a disease that has persisted to contemporary times despite 60 years of anti-leprosy chemotherapy. Chemotherapy has indeed contributed to lowering the global

incidence of leprosy, but the disease still exists. Official reports from 115 countries indicated a global prevalence of 189,018 cases in 2012, with an incidence of 232,857 (new) cases in the same year (WHO 2013). Lepromatous leprosy is the malignant form of the disease, and tuberculoid leprosy is the benign form. Malignancy in lepromatous leprosy correlates with the development of (specific) cellular anergy (Godal *et al.* 1977; Rojas-Espinosa 2007). Although there is a great deal of knowledge on the pathology, bacteriology, immunology and genetic basis of leprosy (Misch *et al.* 2010), the mechanism that leads to anergy in lepromatous leprosy is not completely understood. The lack of a suitable natural model of leprosy has hampered the study of anergy in this disease; intact mice are resistant to human leprosy, and armadillos and certain African monkeys are impractical and costly (Rojas Espinosa & Lovik 2001). However, there is a model of leprosy in the mouse (murine leprosy) in which this phenomenon can be analysed. Murine leprosy is not a counterpart of human leprosy, but it shares with it several characteristics including cellular anergy (Curtis & Turk 1979; Bullock *et al.* 1977; Rojas-Espinosa 2009).

In the present study, we found that *M. lepraemurium* was able to induce an early, yet temporary, cell-mediated immune (CMI) response. This CMI peaked by 2 weeks after inoculation, and it subsequently vanished by week 5 and remained low until week 9 when the experiment was ended. A similar timing of mycobacterial-induced CMI was observed in mice inoculated with *M. bovis* BCG, except that these animals conserved their activated CMI response until the end of the experiment (week 9 postinoculation).

Concomitant to the search for macrophage activation (β-Gal expression), acid-fast bacilli were recorded in the spleens and livers of the MLM- or BCG-inoculated animals. The scarce number of BCG bacilli during the period in which CMI was highest (the first 2 weeks of inoculation) and their absence thereafter indicate that BCG was controlled and eliminated early in the infection by CMI-activated macrophages. In contrast, MLM resisted the effect of the CMI, which also developed within the first 2 weeks of inoculation, and then somehow suppressed it as deduced from the low lymphoproliferative response to MLM, the low levels of β-Gal and nitric oxide, and the increased numbers of bacilli observed thereafter. While the activity of β-Gal is an indicator of overall macrophage activation, the release of nitric oxide is of particular importance because it is noxious to some mycobacteria (Bogdan 2001). Thus, bacteria such as BCG that induce high levels of nitric oxide are prone to succumb to its toxic effects, while bacteria such as MLM that maintain low levels of nitric oxide have a higher probability of remaining alive and causing disease (Rojas-Espinosa *et al.* 2002).

From the above results, we selected an inoculation time of 2 weeks for BCG or MLM, from which potentially active leucocyte extracts (DLE) could be prepared.

Use of DLE for the treatment of illness

Concerning the use of DLE in several clinical anomalies, previous reports have indicated that it is possible to improve patients' immunological condition following the administration of this preparation (Berrón *et al.* 2007; Viza *et al.* 2013). In leprosy, there have been some attempts to ameliorate the clinical pathology of the disease by administering DLE from Mitsuda (lepromin)-positive donors, but the results have been controversial, and no cases have led to the cure of the disease (Bullock *et al.* 1972; Saha *et al.* 1975; Hastings & Job 1978; Faber *et al.* 1979; Leser *et al.* 1980). Although there are many explanations for this outcome, the main explanation is the advanced pathology in lepromatous disease when DLE was administered. Thus, taking advantage of the mouse model of leprosy for which anergy is a characteristic (Rojas-Espinosa 2009), we attempted to control the disease by reversing anergy through the administration of DLE prepared from mice inoculated with nil, BCG or MLM; a control group of mice received a combination of rifampicin and clofazimine, two drugs with a demonstrated anti-leprosy effect. We found that the anti-leprosy drugs had an almost sterilizing effect. In general, DLE in any group, given at a dose of 1.0 U (1×10^6 cells or 3 μg protein) was ineffective but was clearly beneficial when administered at a low dose of 0.1 U. These unexpected results are not easy to explain, but they might be understood assuming the presence of both inducing and inhibitory factors in the DLE, with the inducing factor active at trace concentrations and the inhibitory activity lost by dilution (Lawrence & Borkowsky 1996).

The effectiveness of the treatment was deduced from several observations: (i) a diminution in the size of the dermal lesions, (ii) a reduction in the extension of the granuloma fraction, (iii) a reduction in the number of bacilli within the granulomas and (iv) the development of granulation tissue in and around the lesions. Granulation tissue, which comprises PMN cells, fibroblasts, collagen deposits and new blood vessels, is indicative of a healing process.

The present work challenges the original concept of Lawrence concerning the specificity of DLE because we observed that DLE had a beneficial effect only when administered at low doses (0.1 U, 0.1×10^6 cells, 0.3 μg protein), and their beneficial effect was independent of their supposed specificity. Thus, the substance responsible for the positive effect of DLE must be investigated within the cell metabolites rather than within conventional (specific) immune-related molecules.

Conflict of interest

No conflict of interests declared.

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Authors' contribution

O.R-E involved in the study concept and design. M.J-O participated in data acquisition. O.R-E, M.J-O, V.G.H, P.A-P and E.B-V involved in data interpretation. O.R-E and M.A-S involved in preparation of the manuscript. All authors involved in approval of the manuscript.

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